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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :		(11) International Publication Number: WO 89/12399
A23C 19/11, A61K 37/02	A1	(43) International Publication Date: 28 December 1989 (28.12.89)

(21) International Application Number: PCT/US89/02625

(22) International Filing Date: 16 June 1989 (16.06.89)

(30) Priority data:

209,861	22 June 1988 (22.06.88)	US
317,626	1 March 1989 (01.03.89)	US

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(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), MC, NL (European patent), NO, SE (European patent), SU.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NISIN COMPOSITIONS FOR USE AS ENHANCED, BROAD RANGE BACTERICIDES

(57) Abstract

Bacteriocin compositions comprising lanthionine containing bacteriocins and non-bactericidal agents. When the bacteriocin compositions are combined with a suitable carrier with each component present in sufficient quantities such that the composition is effective against Gram negative bacteria in addition to Gram positive bacteria, they become enhanced, rapid acting, broad range bactericides suitable for a variety of applications.

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Description

Nisin Compositions For Use As Enhanced, Broad Range Bactericides

Background of the Invention

This is a continuation-in-part application of Serial No. 209,861 filed June 22, 1988. Nisin is a polypeptide with antimicrobial properties which is produced in nature by various strains of the bacterium Streptococcus lactis. It is a known food preservative which inhibits the outgrowth of spores of certain species of Gram positive Bacilli.

Although sometimes mistakenly and imprecisely referred to as an antibiotic, nisin is more correctly classified as a bacteriocin, i.e. a proteinaceous substance produced by bacteria and which has antibacterial activity only towards species closely related to the species of its origin. Nisin is a naturally-occurring preservative found in low concentration in milk and cheese, and is believed to be completely non-toxic and non-allergenic to humans.

Nisin has recently been recognized as safe by the FDA as a direct food ingredient in pasteurized cheese spread, pasteurized processed cheese spread, and pasteurized or pasteurized processed cheese spread with fruits, vegetables, or meats. Furthermore, since it is a polypeptide, any nisin residues remaining in foods are quickly digested.

A summary of nisin's properties appears in Hurst, A., Advances in Applied Microbiology 27:85-123 (1981). This publication describes what is generally known about nisin. Nisin, produced by Streptococcus lactis, is available commercially as an impure preparation, Nisaplin™,

from Aplin & Barrett Ltd., Dorset, England and can be obtained by isolating naturally-occurring nisin from cultures of Streptococcus lactis and then concentrating the nisin according to known methods. There are also reported methods for producing nisin using altered strains of Streptococcus. See Gonzalez et al., U.S. Pat. No. 4,716,115, issued December 29, 1987. It should also be possible to produce nisin by recombinant DNA technology.

Nisin has been applied effectively as a preservative in dairy products, such as processed cheese, cream and milk. The use of nisin in processed cheese products has been the subject of recent patents. See U.S. Pat. Nos. 4,584,199 and 4,597,972. The use of nisin to inhibit the growth of certain Gram positive bacteria has been well documented. However, its complete success and acceptance as a food preservative has heretofore been hampered by the belief that nisin was ineffective against Gram negative and many Gram positive bacteria. Gram negative bacteria are almost always present in conjunction with Gram positive bacteria and are a major source of food spoilage and contamination. See Taylor, U.S. Pat. No. 5,584,199, issued April 22, 1986 and Taylor, U.S. Pat. No. 4,597,972, issued July 1, 1986; Tsai and Sandine, "Conjugal Transfer of Nisin Plasmid Genes from Streptococus Lactis 7962 to Leuconostoc Dextranicum 181, Applied and Environmental Microbiology, Feb. 1987, p. 352; "A Natural Preservative," Food Engineering Int'l,, May 1987, pp. 37-38; "Focus on Nisin," Food Manufacture, March 1987, p. 63.

Summary of the Invention

It has now been found that contrary to prior teaching, compositions comprising nisin, in combination with various non-bactericidal agents have enhanced, broad range bactericidal activity against Gram negative bacteria as well as enhanced activity against a broader range of Gram posi-

tive bacteria than nisin alone. The enhanced bactericidal activity against Gram positive bacteria occurs in a pH range broader than previously taught. The invention provides bacteriocin compositions of nisin or other, lanthionine containing bacteriocins, in combination with various non-bactericidal agents for example chelating agents or surfactants. The invention further provides the compositions dissolved or suspended in a suitable carrier to yield enhanced broad range bactericides.

Detailed Description of the Invention

Specifically, it has been found that a solution of about 0.1 μg/ml to 300 μg/ml of nisin in the presence of about 0.1 mM to 20 mM of a chelating agent, for example EDTA, virtually eliminates the growth of Gram negative bacteria such as Salmonella typhimurium, Escherichia coli, Pseudomonas aeruginosa, Bacterioides gingivalis, Actinobacillus actinomycetescomitans, and Klebsiella pneumoniae and is more active towards Gram positive bacteria such as Staphylococcus aureus, Streptococcus mutans, Listeria monocytogenes Streptococcus agalactiae and Coryneform bacteria than nisin alone. Although the enhancement of nisin activity by chelator was concentration dependent, contrary to expectations, concentrations of EDTA in excess of 20mM were inhibitory to the bactericidal activity of nisin. However, in the presence of a proteinaceous carrier, and polyvalent polymers such as serum albumin, collagen, gelatin, casein and keratin, the inhibition of nisin by concentrations of EDTA above 20mM was significantly reduced, thereby extending the useful range of EDTA enhancement of nisin.

It has also been found that a solution of about 0.1 μg/ml to 300 μg/ml nisin and about 0.1 mM to 20 mM of a chelating agent will further enhance the effectiveness of nisin against Gram negative and Gram positive bacteria in

the presence of about 0.01% to 1.0% of surfactant. Additionally, it has been found that, in the presence of surfactant alone, nisin has enhanced activity against Gram positive bacteria.

In the present invention, suitable chelating agents include, but are not limited to, EDTA, CaEDTA, $CaNa_2EDTA$, and other alkyldiamine tetraacetates, EGTA and citrate. Surfactants, valuable as cleansing agents, suitable for combination with nisin, with or without EDTA, include, but are not limited to, the nonionic surfactants Tweens, Tritons, and glycerides, ionic surfactants such as fatty acids, quaternary compounds, anionic surfactants such as sodium dodecyl sulphate and amphoteric surfactants such as cocamidopropyl betaine and emulsifiers.

Since Gram positive and Gram negative bacteria are almost always found together in foods, the effectiveness of the nisin compositions towards Gram negative bacteria such as Salmonella typhimurium, Escherichia Coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacterioides gingivalis, Actinobacillus actinomycetescomitans, and other Gram negative pathogens and Gram positive bacteria will be of great use. The bactericides are particularly suited for the control and prevention of contamination of raw ingredients, processed foods and beverages by bacterial pathogens and other microbial spoilage organisms. Potential food related uses include treatment of meats, especially poultry, eggs, cheese and fish and treatment of food packaging and handling equipment. Further uses include as food preservative, such as in processed cheese, cream, milk, dairy products and in cleaning poultry, fish, meats, vegetables, and dairy and food processing equipment. The use of the nisin compositions should not be limited to food related uses and the nisin compositions should be useful in any situation in which there is a need or desire to eliminate Gram negative and Gram positive bacteria.

The compositions can be dissolved in a suitable carrier for example an aqueous solvent or buffer or suspended in any suitable liquid, colloidal or polymeric matrix to create bactericides. The compositions or bactericides can be incorporated into ointments or coatings for medicinal uses such as the treatment of infections, wound dressings or surgical implants and as a broad spectrum disinfectant for skin or oral rinses, disinfectant scrubs, wipes or lotions. The bactericides can be used for cleaning medical instruments, in pre-operative surgical scrubs and the like. The bactericides are particularly useful in circumstances where environmental disinfection is desired but where chemical germicidals are precluded because of the risks of corrosive or otherwise toxic residues.

Unlike the activity of most broad spectrum germicidals which is compromised by the presence of complex organic matter, the compositions of the present invention are effective as bactericides in the presence of organic matter, such as milk or serum.

Nisin was known to optimally inhibit the growth of a few closely related Gram positive bacteria, particularly certain Gram positive spore forming bacilli at pH 5.0. The bactericidal activity of nisin in solution with a chelating agent was surprisingly rapid and greatly enhanced towards a broad range of Gram positive bacteria at pH values greater than pH 5.0, and, moreover, was activated towards Gram negative bacteria at both acidic and basic pH, preferably in the range pH 5.0 to 8.0. This unexpectedly rapid and broad-ranged bactericidal activity of chelator-activated nisin makes it suitable for use as, among other things, a disinfectant.

Nisin belongs to the class of peptide bacteriocins containing lanthionine. Also included among that class are subtilin, epidermin, cinnamycin, duramycin, ancovenin and Pep 5. These bacteriocin peptides are each produced by

different microorganisms. However, subtilin obtained from certain cultures of Bacillus subtilis, and epidermin obtained from certain cultures of Staphylococcus epidermidis, have been found to have molecular structures very similar to that of nisin (see Hurst, pp. 85-86, and Schnell et al., Nature, 333:276-278). It is therefore believed that because of the molecular similarities, other lanthionine containing peptide bacteriocins will be equally as effective as nisin in combination with chelating agents and non-ionic surfactants in eliminating Gram negative and Gram positive bacterial contaminations.

The effectiveness of the nisin, and by extension other lanthionine containing peptide bacteriocin, compositions as bactericides against Gram negative bacteria is surprising, since the prior art generally teaches away from this activity of nisin. The enhanced activity of nisin against Gram positive bacteria in the presence of EDTA at a pH greater than 5.0 is unexpected since it was previously believed that nisin activity is optimal at pH 5.0. Furthermore, the discovery of such effectiveness of the nisin and lanthionine containing peptide bacteriocin compositions as bactericides fulfills a long-felt need in the science of food preservation, which has suffered from the absence of an acceptable, natural, non-toxic agent effective against a broad range of bacteria.

In order to demonstrate the superior and unexpected rapid activity of the composition containing nisin, EDTA and/or various surfactants against both Gram negative and Gram positive bacteria, a number of experiments were conducted with the bactericides. These experiments are meant as illustration and are not intended to limit this invention. It is to be expected that other, lanthionine containing peptide bacteriocins would be effective substitutes for nisin and that chelating agents other than EDTA will be effective substitutes for EDTA.

All tests in the following examples were performed at 37°C. The efficacy of the enhanced broad range bactericides was determined by assaying bactericidal activity as measured by the percent bacterial survival after treatment with the bactericide. Generally, after incubation of a 10^7 cell per ml suspension of target species with the novel bactericide for specified lengths of time, bacteria were collected by centrifugation for 2 minutes. The bacterial pellet was washed free of the bactericide with a rescue buffer, termed herein Phage buffer (50mM Tris-HCl buffer pH 7.8, 1mM $MgSO_4$, 4mM $CaCl_2$, 0.1 M Nacl, and 0.1% gelatin), resuspended and serially diluted into Phage buffer, and 100ml of the suspended bacteria were spread on nutrient agar plates. Surviving bacteria were determined by scoring colony forming units (CFU) after incubation for 24-48 hours at 37°C. An effective bactericide according to this invention is one which allows less than 0.1% of the initial viable count of the bacteria to survive.

Example 1

Activity of Nisin and a Chelating Agent Against Gram Negative Bacteria (S. typhimurium)

As shown in Table 1, two tests were conducted in 20mM Tris, pH 8.0 at 37°C to show the effect of the bactericide containing nisin and the chelating agent EDTA alone. Test #1, a control, was conducted without EDTA and shows the effect of nisin alone toward the Gram negative bacterium S. typhimurium. The increased concentrations of nisin do exhibit some activity, but even the activity of the higher concentrations in the absence of EDTA, 1.6% survival at 100 μg/ml nisin, is wholly inadequate for a food preservative. The level of bactericidal activity obtained from nisin and EDTA is significant.

TABLE 1

Test #	Initial Viable Bacteria Count	EDTA (mM)	Nisin (μg/ml) 0	10	30	50	100	300
			Percentage S. typhimurium Survival at 3 hours					
1	3.0×10^6	0	100	51.3	-	7.0	1.6	-
2	5.7×10^6	20	2.5	-	10^{-3}	-	$<10^{-4}$	$<10^{-4}$

Test #2 (Table 1), conducted using nisin plus 20mM EDTA, demonstrates the surprising activity of the nisin composition in eliminating the target Gram negative bacteria.

Table 1 shows that in test #2 at a concentration of 20mM EDTA and 30 μg/ml of nisin, the bactericide has a marked bactericidal activity towards S. typhimurium, while at nisin concentrations of 100μg/ml and greater, the nisin and EDTA bactericide virtually eliminates the bacteria (percentage survival less than 10^{-4} which indicates no surviving bacteria in the assay). Thus, the combination of EDTA and nisin demonstrates a synergistic activity of greater than 1000 times that of nisin alone.

Example 2

Activity of Nisin, a Chelating Agent and a Surfactant Against Gram Negative Bacteria (S. typhimurium)

Four tests (Table 2) were conducted to determine the effect on S. typhimurium of the bactericide containing nisin and both EDTA and the surfactant Triton X-100 in 20mM Tris, pH 8.0 at 37°. The control (Test #1) is identical to the control of Example 1 (Table 1).

TABLE 2

Test #	Initial Viable Bacteria Count	EDTA (mM)	Triton X-100 (%)	Nisin (g/ml) 0	10	30	50	100	300
				Percentage S. typhimurium Survival at 3 hours					
1	3.0×10^6	0	0	100	51.3	-	7.0	1.6	-
2	3.0×10^6	0	1.0	37.4	93.0	-	64.0	47.0	-
3	5.7×10^6	20	0.1	0.03	-	$< 10^{-3}$	-	-	-
4	5.7×10^6	20	1.0	$< 10^{-4}$	-	$< 10^{-4}$	-	$< 10^{-4}$	$< 10^{-4}$

Test #2 (Table 2) was conducted using nisin and 1.0% Triton X-100, but without EDTA. The presence of the detergent alone inhibits the activity of the nisin towards the Gram negative bacteria and nisin was ineffective. However, in tests #3 and #4 (Table 2), which represent the invention, the presence of 20mM EDTA in combination with Triton X-100 is a bactericide which markedly increases the bactericidal activity of nisin towards S. typhimurium. Indeed the combination of Triton X-100 with EDTA but without nisin was effective, although to a lesser degree than in the presence of nisin. While in both tests #3 and #4 (Table 2) the nisin combinations were very effective, the concentration of 1.0% Triton X-100 (test #4, Table 2) was most effective.

The presence of the non-ionic surfactant, Triton X-100, in combination with EDTA, enhances the activity of nisin toward Gram negative bacteria even more than the bactericide containing nisin and EDTA alone (Example 1).

Example 3

Activity of Nisin, a Chelating Agent and a Surfactant Against Gram Negative Bacteria (S. typhimurium)

Table 3 shows the enhanced activity toward S. typhimurium of the bactericide containing nisin, 20mM of the chelating agent EDTA and the non-ionic surfactant Tween 20 in 20 mM Tris, pH 8.0 at 37°C. As with Triton X-100 (Example 2) the combination of nisin and EDTA with (1%) of Tween 20 is most effective.

TABLE 3

Test #	Initial Viable Bacteria Count	EDTA (mM)	Tween20 (%)	Nisin (μg/ml) 0	10	30	50	100	300
				Percentage S. typhimurium Survival at 3 hours					
1	3.0×10^6	0	0	100	51.3	-	7.0	1.6	-
2	5.7×10^6	20	0	2.5	-	$< 10^{-3}$	$- < 10^{-4}$		$< 10^{-4}$
3	4.3×10^6	20	1.0	$< 10^{-2}$	-	$< 10^{-4}$	$- < 10^{-4}$		$< 10^{-4}$

Example 4

Activity of Nisin, a Chelating Agent and a Surfactant Against Gram Negative Bacteria (Escherichia coli)

The effect of the bactericide containing nisin and EDTA towards the Gram negative bacteria E. coli was demonstrated, as shown in Table 4.

TABLE 4

Test #	Initial Viable Bacteria Count	Triton EDTA (mM)	X-100 (%)	Nisin (μg/ml) 0	30	100	300
				Percentage E. coli Survival at 2 hours			
1	1.0×10^7	0	0	100	27	25	8.5
2	1.0×10^7	20	0	14.5	0.86	0.01	0.001
3	1.0×10^7	0	1.0	100	-	30	-
4	1.0×10^7	20	1.0	1.2	0.8	0.05	$<10^{-4}$

The tests, with and without EDTA, were performed in 20mM Tris buffer solution, pH 8.0 at 37°C, with an initial viable count of 1×10^7 E. coli cells/ml. The effects of the bactericide were measured as a function of percentage bacteria survival after 2 hours.

In test #1, (control, Table 4) without EDTA, nisin exhibited little meaningful activity toward the elimination of E. coli. In test #2 (Table 4), however, where 20mM EDTA was present, the bactericidal composition exhibited substantial activity towards the E. coli bacteria. The activity increased in effectiveness as the concentration of nisin was increased. The combination of nisin with EDTA as a bactericide demonstrates a 1000 fold synergistic increase in effectiveness towards E. coli. In tests #3 and #4 (Table 4), it can be seen that Triton X-100 has no significant bactericidal activity towards E. coli. In fact, Triton X-100 appears to inhibit nisin activity towards Gram negative bacteria as was found with S. typhimurium (Table 2). However, the overall enhancement of nisin by EDTA substantially reverses the inhibitory effects of Triton X-100 as seen in Tables 2 and 4.

It thus appears that the bactericide containing nisin and a chelating agent, such as EDTA, is an effective food preservative towards various types of Gram negative bacteria even in the presence of surfactants.

Example 5

Activity of Nisin and a Chelating Agent Against Gram Negative Bacteria (Klebsiella pneumoniae)

The effect of the bactericide containing nisin and EDTA alone towards the Gram negative bacteria K. pneumoniae was demonstrated, as shown in Table 5.

TABLE 5

Test	Initial Viable Bacteria Count	EDTA (mM)	Triton X-100 (%)	Nisin μg/ml 0	30	100	300
				% Survival at 2 hours			
1	10^7	0	0	100	-	50	38
2	10^7	20	0	22	0.5	1.1	0.085

The two tests, one with and one without EDTA (control), were performed in 20 mM Tris buffer, pH 8.0 at 37°C with an initial viable count of 10^7 cells/ml of K. pneumoniae. The effect was measured as a function of percentage bacterial survival after 2 hours.

In test #1, (control, Table 5) without EDTA, nisin exhibited little meaningful bactericidal activity toward K. pneumoniae. In test #2 (Table 5), however, where 20mM EDTA was present, the bactericide exhibited substantial activity towards K. pneumoniae. The activity increased in effectiveness as the concentration of nisin was increased.

Example 6

Nisin Activity Against Gram Negative Bacteria (*Salmonella typhimurium*) is Dependent on Chelator Concentration

The data in Table 6 demonstrate that the enhanced activation of nisin towards Gram negative bacteria (*S. typhimurium*) is dependent on the concentration of EDTA in either 50 mM sodium acetate, pH 5.0, or 20 mM Tris, pH 8.0 at 37°C.

TABLE 6

Test #	pH	Initial Viable Bacterial Count	Nisin μg/ml	EDTA(mM) 0	0.2	2.0	10	50	100
				% Survival at 2 hours					
1	5.0	$3x10^6$	0	100	-	38.7	15.2	3.5	-
2	5.0	$3x10^6$	100	0.6	10^{-4}	10^{-4}	0.004	0.02	-
3	8.0	$5x10^6$	0	100	-	8.7	14	11.4	45
4	8.0	$5x10^6$	100	4	10^{-4}	10^{-4}	10^{-4}	0.6	30

In tests #1 and #3, (controls, Table 6) using EDTA concentrations up to 100 mM without nisin exhibited little meaningful activity towards S. typhimurium at either pH 5.0 (#1) or pH 8.0 (#3). In tests #2 and #4 (Table 6), however, where 100 μg/ml nisin was present in combination with EDTA, the bactericides exhibited substantial activity towards S. typhimurium. The activity of the bactericides was similar at both acidic pH (5.0) and basic pH (8.0), despite the fact that the activity of nisin alone towards Gram positive bacteria is optimal at pH 5.0.

The enhancement of nisin by EDTA was concentration dependent, being optimal in the range 0.2 mM to 10 mM at pH values 5.0 and 8.0. Surprisingly, at concentrations greater than 10 mM EDTA, the enhancement of nisin by EDTA becomes reduced; the reduction of activation is significantly greater at pH 8.0 than at pH 5.0.

Example 7

Nisin and a Chelating Agent Against Gram Negative Bacteria (S. typhimurium)

The enhancement of the activity of nisin by EDTA towards Gram negative bacteria in the presence of biological tissue was demonstrated with S. typhimurium on chicken muscle, and is shown in Table 7.

TABLE 7

Test #	pH	Nisin µg/ml	EDTA(mM) 0	0.1	0.3	1	3	10	20	30	100
			% Survival[a] at 2 hours								
1	5.0	0	11.8	-	-	-	-	6.4	-	-	-
2	5.0	300	0.1	0.2	0.05	0.01	0.003	0.016	0.03	0.02	0.07
3	8.0	0	100	-	-	-	-	5.2	-	-	-
4	8.0	300	7.5	0.1	0.02	0.02	0.09	0.47	0.5	-	2.2
5	8.0	300[b]	0.02	0.09	0.0002	$<10^{-4}$	0.0004	0.003	-	0.03	0.09

[a] Unadhered cells

[b] Contains 1% Bovine serum albumin (BSA)

Incubations were performed in either 50mM sodium acetate, pH 5.0, or 20 mM Tris, pH 8.0 at 37°C.

Cubes of chicken muscle were cleansed with sodium hypochlorite and povidone iodine prior to use. To inoculate the tissue, the cubes of chicken muscle were dipped into a 10^8 cells/ml suspension of S. typhimurium in 20 mM Tris HCl, pH 8.0. Excess moisture was removed from dipped cubes by tapping. The chicken samples were placed into sufficient buffer containing the nisin composition to cover the tissue and incubated for 2 hours at 37°C after which the tissue was removed to sufficient Phage buffer to cover the tissue. The bacteria remaining in the test solution were collected by centrifugation, washed with Phage buffer, and combined with bacteria washed from the tissue by Phage buffer. The combined samples (termed "unadhered" cells) were serially diluted and 100 µl aliquots were plated for determination of surviving bacteria.

In tests #1 and #3 (Table 7), in the absence of nisin at either pH 5 or pH 8, EDTA alone has no significant effect on the survival of S. typhimurium. In tests #2 and #4 (Table 7), however, where 300 µg/ml nisin was present, the bactericides exhibited substantial activity towards S. typhimurium on chicken muscle at both pH 5.0 and pH 8.0.

The enhancement of nisin by EDTA was concentration dependent, the optimal concentration being in the range 0.3mM to 10mM EDTA at both pH values 5.0 and 8.0. At concentrations greater than 10mM EDTA at pH 8.0, the activation of nisin by EDTA is reduced. However, as is shown in test #5 (Table 7), in the presence of 1.0 % bovine serum albumin at pH 8.0, the efficacy of nisin towards S. typhimurium on chicken muscle is expressed throughout the range of EDTA concentrations up to 100mM.

Thus, bactericides containing nisin and low concentrations of chelating agent, such as EDTA in the range

0.1mM to 20mM, can be extremely effective for the elimination or prevention of contamination of food by Gram negative bacteria.

Example 8

Titration of Nisin Activity Against Gram Negative Bacteria (S. typhimurium)

At the optimal concentration of chelating agent, the efficacy of the bactericide in Tris buffer towards Gram negative bacteria was demonstrated to be substantial, as is shown in Table 8.

TABLE 8

Test #	Initial Viable Bacterial Count	EDTA (mM)	BSA %	% Survival at 2 hours Nisin μg/ml 0	0.1	0.3	1.0	3.0	10	30	100
1	$6x10^6$	0	0	100	-	-	-	-	51.3	-	1.6
2	$6x10^6$	1.0	1.0	63	0.7	0.08	0.01	0.05	0.01	$<10^{-4}$	-

In test #2 (Table 8), it can be seen that as little as 0.3 μg/ml of nisin, with 1.0 mM EDTA in 20mM Tris at pH 8.0 in the presence of 1% bovine serum albumin (BSA), significantly reduced the survival of S. typhimurium. The bactericide is as active towards Gram negative bacteria as nisin alone is towards Gram positive Streptococci.

Example 9

Titration of Nisin Activity Against Gram Negative Bacteria (S. typhimurium)

At the optimal concentration of chelating agent, the efficacy of a bactericide towards Gram negative bacteria in the presence of biological tissue was demonstrated with S. typhimurium on chicken muscle, and is shown in Table 9.

TABLE 9

Test #	pH	Initial Viable Bacterial Count	EDTA (mM)	BSA (%)	Nisin μg/ml 0	10	100	200	300
					% Survival at 2 hours				
1	8.0	$3x10^7$	0	0	100	-	-	-	-
2	8.0	$3x10^7$	1.0	1.0	27	0.26	0.008	0.007	0.006

Cubes of chicken muscle were cleansed with sodium hypochlorite and povidone iodine prior to use. To inoculate the tissue, the cubes of chicken muscle were dipped into a 10^8 cells/ml suspension of S. typhimurium in 20 mM Tris HCl, pH 8.0. Excess moisture was removed from dipped cubes by tapping. The tissue was placed into sufficient buffer containing the nisin compositions to cover the tissue, and incubated for 2 hours at 37°C after which the tissue was removed to sufficient Phage buffer to cover the tissue. The bacteria remaining in the test solution were collected by

centrifugation, washed with Phage buffer, and combined with bacteria washed from the tissue by Phage buffer. The combined samples (termed "unadhered" cells) were serially diluted and 100µl aliquots were plated for determination of surviving bacteria.

Example 10

Nisin EDTA and Methyl Paraben Activity Against Gram Negative Bacteria (S. typhimurium)

A bactericide containing nisin and EDTA, when combined with a known food preservative, methyl paraben, was demonstrated to be exceptionally effective towards Gram negative bacteria, as shown in Table 10.

TABLE 10

Test #	Initial Viable Bacterial Count	Nisin g/ml	EDTA[b] (mM)	% Methyl Paraben 0	0.1	1.0
				% Survival[c] at 2 hours		
1	$3x10^6$	0	10	11.8	1.0	10^{-4}
2	$3x10^6$	300	10	0.03	$<10^{-3}$	$<10^{-4}$

[b] 50 mM Na acetate buffer, pH 5.0

[c] Unadhered cells

Cubes of chicken muscle were cleaned with sodium hypochlorite and povidone iodine prior to use. To inoculate the tissue, the cubes of chicken muscle were dipped into a 10^8 cells/ml suspension of S. typhimurium in 50 mM sodium acetate buffer, pH 5.0. Excess moisture was removed from dipped cubes by tapping. The tissue was placed into sufficient buffer containing nisin compositions to cover the tissue, and incubated for 2 hours at 37°C after which the

tissue was removed to sufficient Phage buffer to cover the tissue. The bacteria remaining in the test solution were collected by centrifugation, washed with Phage buffer, and combined with bacteria washed from the tissue by Phage buffer. The combined samples (termed "unadhered" cells) were serially diluted and 100 µl aliquots were plated for determination of surviving bacteria.

In test #1 (Table 10), methyl paraben in the presence of 10 mM EDTA was shown to be effective towards S. typhimurium only at a concentration of 1.0%. In test #2 (Table 10), however, in the presence of 300 µg/ml nisin, the effectiveness of methyl paraben and nisin towards S. typhimurium was substantially improved.

The compositions containing nisin and EDTA significantly improve the utility of the food preservative methyl paraben. Furthermore, the bactericides may lead to substantial reductions in the concentrations, or eliminate the need for these commonly recognized, though less desirable, food preservatives such as methyl paraben.

Example 11

Nisin and Chelating Agent Activity Against Gram Positive Bacteria (Staphylococcus aureus)

The activation of nisin by a chelating agent is pH-dependent. The data in Table 11 confirm that at pH 5.0, nisin is somewhat more bactericidal towards S. aureus than is nisin at pH 8.0. At pH 5.0, EDTA does not enhance nisin activity towards S. aureus and at concentrations of EDTA greater than 10 mM, EDTA is inhibitory to the bactericidal activity of nisin. However, the bactericidal activity of nisin activated by EDTA at pH 8.0 is significantly greater than the bactericidal activity of nisin alone, or in combination with EDTA at pH 5.0.

Table 11

Influence of pH on the Effects of EDTA on Nisin Bactericidal Activity towards Staphyloccus aureus

pH	Nisin μg/ml	EDTA mM 0	0.1	0.3	1.0	3.0	10	30	100
		% Survival 2 hr[a]							
8.0	0	100	-	100	81	100	100	100	-
8.0	3.0	7.4	0.03	0.01	0.2	0.4	3	56	-
5.0	0	100	-	-	-	100	-	-	-
5.0	3.0	0.6	1.0	1.3	1.4	1.8	-	34	80

[a] Initial viable count: 8.0×10^6 cfu/ml

Incubations were performed in 50 mM sodium acetate buffer, pH 5.0 or 20 mM Tris-HCl buffer, pH 8.0 at 37°C.

The bactericidal activity of nisin alone is reported (see Hurst) to be greatest at pH 5.0 or lower, and data presented in Table 11 support this. On the basis of this information it was believed that the bactericidal activation of nisin by EDTA towards S. aureus would likewise be greatest at lower pH. However, as can be seen in Table 11 and contrary to expectations (see Table 6), EDTA was not observed to enhance nisin activity towards Gram positive bacteria at pH 5.0. However, inhibition of nisin activity by high concentrations of EDTA was still observed at pH 5.0. Thus, the activation of nisin by a chelating agent occurs only within a range of chelator concentrations and, with respect to Gram positive bacteria, is dependent upon pH with the preferred pH range greater than pH 5.0.

Example 12

Nisin and Chelating Agent Activity Against Gram Positive Bacteria

The effects of EDTA on the bactericidal activity of nisin at pH 8.0 are not limited to S. aureus, an important human pathogen, but are also observed with Streptococcus mutans, responsible for dental plaque (Table 12A), Listeria monocytogenes, a foodborne pathogen (Table 12B), and with a mixed population of axillary Coryneform bacteria, contributors to body odor (Table 12C).

Table 12A

The Effects of EDTA on the Bactericidal Activity of Nisin towards Streptococcus mutans

pH	Nisin μg/ml	EDTA mM 0	0.01	0.1	0.3	1.0	3.0	10	30	100
		% Survival after 2 hr[a]								
8.0	0	100	-	-	-	-	-	-	-	-
8.0	0.1	4.3	1.8	0.04	0.02	0.06	1	25	100	100

[a] Initial viable count: 6.0×10^6 cfu/ml

Incubations were performed in 20 mM Tris-HCl, pH 8.0 at 37°C.

Table 12B

The Effects of EDTA on the Bactericidal Activity towards Listeria monocytogenes

pH	Nisin μg/ml	EDTA mM 0	0.1	0.3	1.0	3.0	10	30	100
		% Survival after 2hr[a]							
8.0	0	100	-	-	84	-	-	-	-
8.0	3.0	0.71	0.04	0.04	0.02	0.1	0.64	10	14

[a] Initial viable count: 6.0×10^6 cfu/ml

Incubations were performed in 20 mM Tris-HCl, pH 8.0 at 37°C.

Table 12C

The Effects of EDTA on Nisin Bactericidal Activity towards Coryneform bacteria

pH	Nisin g/ml	EDTA mM 0	0.1	0.3	1.0	3.0	10
		% Survival 2hr[a]					
8.0	0	100	-	4.6	3.6	8	36
8.0	3	0.22	0.03	0.0009	0.1	--	0.16

[a] Initial viable count: 1.0×10^6 cfu/ml

Incubations were performed in 20 mM Tris-HCl, pH 8.0 at 37°C.

Example 13

Rapid Bactericidal Activity of Nisin Activated by Chelator

The bactericide comprising nisin with EDTA is rapidly bactericidal as is illustrated by the data presented in Table 13A. Suspensions of the Gram positive bacterium S. mutans at 10^7 cells/ml were incubated in 20 mM Tris buffer, pH 7.3 at 37°C with a range of concentrations of nisin activated by 1 mM EDTA. The suspensions were incubated for various times ranging from 0.5 to 60 minutes with the bactericides. The bactericidal efficacy of the bactericides was estimated by determining the percent survival of bacteria. Enhanced by EDTA, as little as 10 μg/ml of the nisin in this formulation is able to reduce the bacterial load by 6 logs within 1 minute.

Rapid bactericidal activity is a prerequisite for effective disinfection. Thus, the compositions are predicted to be effective bactericides particularly as demonstrated here, as a component of a mouthwash, rinse, toothpaste, or other similar dentrifice active against plaque forming S. mutans.

The activity of nisin enhanced by EDTA against Gram negative bacteria after 2-3 hours was shown in Examples 1-7. Rapid bactericidal activity of nisin enhanced by EDTA is also seen towards Gram negative bacteria and this is illustrated by the data in Table 13B.

Table 13A

Kinetics of Bactericidal Activity towards Streptococcus mutans of Nisin Enhanced by EDTA

Incubation Time (Minutes)	Nisin μg/ml with 1.0 mM EDTA					
	0	1	3	10	30	100
	% Survival[a]					
0.5	-	-	-	-	-	$<10^{-4}$
1	-	-	-	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
3	100	0.5	0.002	$<10^{-4}$	$<10^{-4}$	-
15	-	0.03	$<10^{-4}$	$<10^{-4}$	-	-
30	-	-	$<10^{-4}$	-	-	-
60	100	0.003	-	-	-	-

[a] Control viable cell count: 1.0×10^7 cfu/ml

Incubations were performed in 20 mM Tris-HCl, pH 7.3 at 37°C.

TABLE 13B

Rapid Bactericidal Activity towards Escherichia coli of Nisin Enhanced by EDTA

	Nisin 4g/ml						
mM EDTA	0	0.3	1.0	3	10	30	100
	% survival at 1 min[a]						
1.0	100	100	56	0.37	0.013	0.015	0.008

[a] Initial viable count: 1.0×10^7 cfu/ml

Incubations were performed in 20 mM Tris, pH 7.0 at 37°C.

Example 14

Effect of Divalent Cations on EDTA Enhancement of Nisin Activity

Divalent cations bind to EDTA and other chelating agents and would be expected to neutralize the activation of nisin by EDTA. However, as can be seen by the data in Table 14, the bactericidal activity of nisin against S. mutans is enhanced by 1 mM EDTA even in the presence of 1 mM Ca^{2+} ion; only above 3 mM was Ca^{2+} ion inhibitory to EDTA-activated nisin. This is particularly important in mouthwash applications where calcium ion concentrations are relevant.

TABLE 14

Rapid Bactericidal Activity towards Streptococcus mutans of Nisin Activated by EDTA in the presence of Divalent Cation

Nisin	$CaCl_3$ mM 0	0.1	0.3	1.0	3	10
	% survival at 1 min.[a]					
0	100					
3	2.9					
3^E	0.0042	0.0042	0.052			18
30^E	0.0019		0.0003	0.0004	0.06	6.8
100^E	$<10^{-4}$		$<10^{-4}$	$<10^{-4}$	0.0001	1.5

E 1 mM Na_2EDTA

a Initial viable count 1.0 X 10^2 cfu/ml.

Incubations performed in 10% Fetal Calf Serum at 37°C.

Example 15

Nisin and Surfactant Activity Against Gram Positive Bacteria

The bactericidal activity of nisin can also be significantly enhanced when combined with a surfactant alone. This is best illustrated at a limiting nisin concentration (0.2 μg/ml) as shown in Table 15A. At concentrations up to 0.1%, the food grade surfactant monolaurin has little significant bactericidal activity towards Streptococcus agalactiae in the complex medium milk. Nisin, at concentrations up to 0.2 μg/ml, likewise does not

exhibit significant bactericidal activity in milk. However, the combination of the two agents, 0.1% monolaurin and nisin 0.2 g/ml, is extremely potent towards S. agalactiae. This bactericide is over 100 times more active than what would be expected for the additive effect and 10,000 times more active than either of the components individually. Thus, when the application of nisin is limited by its available activity, a bactericide comprising nisin with a surfactant can be expected to be more useful.

An example of where the application of nisin is limited by its available activity is illustrated by the data in Table 15B. Although nisin, and particularly the bactericide comprising nisin and EDTA, is bactericidal towards L. monocytogenes, the data in Table 15B demonstrate that in a complex medium like milk the available nisin activity towards this organism is restricted. However, the bactericide comprised of nisin with the glyceride, monooleate, is effective in milk towards this foodborne pathogen even though monooleate by itself had no bactericidal activity towards this organism.

Table 15A

Nisin Bactericidal Activity towards Streptococcus agalactiae in milk at 37°C

(Activation of nisin by monolaurin)

Nisin (μg/ml)	Monolaurin (%) 0	0.01	0.1
	% survival at 2h[a]		
0	100	100	4.5
0.02	100	100	0.2
0.2	2.2	0.05	0.0008

[a] Initial visable counts 6.0 X 10^7 cfu/ml.

Incubations were in milk at 37°C.

Table 15B

Nisin Bactericidal Activity towards *Listeria monocytogenes* in milk at 37°C

(Activation of nisin by monooleate)

Nisin μg/ml	% Monooleate 0	0.1	1.0
	% Survival 2 hr[a]		
0	100	67	63
100	0.56	10^{-3}	10^{-4}

[a] Initial viable count 5.0×10^7 cfu/ml

Incubations were in milk at 37°C.

Claims

1. A composition comprising a lanthionine containing bacteriocin and a chelating agent.

2. A composition comprising a lanthionine containing bacteriocin and a surfactant.

3. A composition comprising a lanthionine containing bacteriocin, a chelating agent and a surfactant.

4. The composition as defined in claim 1, 2 or 3 wherein the lanthionine containing bacteriocin is selected from the group consisting of nisin, subtilin, epidermin, cinnamycin, duramycin, ancovenin and Pep 5.

5. The composition as defined in claim 1 or 3 wherein the chelating agent is selected from the group consisting of alkyldiamine tetraacetates, CaEDTA, $Na_2CaEDTA$, EGTA and citrate.

6. The composition as defined in claim 5 wherein the alkyldiamine tetraacetate is EDTA and the bacteriocin is nisin.

7. The composition as defined in claim 2 or 3 wherein the surfactant is selected from the group consisting of Tritons, Tweens, glycerides, fatty acids, emulsifiers, quaternary compounds, amphoteric and anionic surfactants.

8. The composition as defined in claim 1 also containing a food perservative.

9. An enhanced broad range bactericide comprising a carrier, a lanthionine containing bacteriocin and a chelating agent.

10. An enhanced broad range bactericide comprising a carrier and a lanthionine containing bacteriocin and a surfactant.

11. An enhanced broad range bactericide comprising a carrier, a lanthionine containing bacteriocin, a chelating agent and a surfactant.

12. The enhanced broad range bactericide as in claim 9, 10 or 11 wherein the lanthionine containing bacteriocin selected from the group consisting of nisin, subtilin, epidermin, cinnamycin, duramycin, ancovenin and Pep 5 and the chelating agent selected from the group consisting of alkyldiamine tetraacetates, EGTA and citrate are present in quantities such that the bactericide has enhanced effectiveness against at least one of the bacteria from the group consisting of Staphylococcus aureus, Streptococcus mutans, Listeria monocytogenes, Streptococcus agalactiae, Cornyeform bacteria, Salmonella typhimurium, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacterioides gingivalis and Actinobacillus actinomycetescomitans.

13. The enhanced broad range bactericide as in claim 12 wherein the alkyldiamine tetraacetate is EDTA.

14. The enhanced broad range bactericide as in claim 10 or 11 wherein the surfactant is selected from the group consisting of Tritons, Tweens, glycerides, fatty acids, quaternary compounds, emulsifiers, amphoteric and anionic surfactants and is present in an amount sufficient such that the bactericide has enhanced effectiveness against at least one of the bacteria from the group consisting of Gram negative and Gram positive bacteria.

15. The enhanced broad range bactericide as in claim 12 wherein the concentration of nisin is between about 0.1 μg/ml and 300.0 μg/ml and the concentration of chelating agent is between about 0.1 mM and 20mM.

16. The enhanced broad range bactericide as in claim 14 wherein the concentration of surfactant is between about 0.01% and 1.0%.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/02625

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: A 23 C 19/11, A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	A 23C, A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	GB, A, 738655 (APLIN & BARRETT LTD) 19 October 1955, see page 2, line 115 - page 3, line 28 --	1-16
A	Chemical Abstracts, vol. 86, no. 1, 3 January 1977, (Columbus, Ohio, US), A.I. Pedenko et al.: "Effect of the antibiotic nisin on pathogenic staphylococci and streptococci" see page 58, abstract no. 594x & Tr. S'ezda Mikrobiol. Ukr. 4th 1975, 221-2	1-16

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* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
2nd October 1989	24 OCT 1989
International Searching Authority	**Signature of Authorized Officer**
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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8902625 SA 29530

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 18/10/89 The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB-A- 738655		None	

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :		(11) International Publication Number: WO 89/12399
A23C 19/11, A61K 37/02	A1	(43) International Publication Date: 28 December 1989 (28.12.89)

(21) International Application Number: PCT/US89/02625

(22) International Filing Date: 16 June 1989 (16.06.89)

(30) Priority data:

209,861	22 June 1988 (22.06.88)	US
317,626	1 March 1989 (01.03.89)	US

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(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), MC, NL (European patent), NO, SE (European patent), SU.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NISIN COMPOSITIONS FOR USE AS ENHANCED, BROAD RANGE BACTERICIDES

(57) Abstract

Bacteriocin compositions comprising lanthionine containing bacteriocins and non-bactericidal agents. When the bacteriocin compositions are combined with a suitable carrier with each component present in sufficient quantities such that the composition is effective against Gram negative bacteria in addition to Gram positive bacteria, they become enhanced, rapid acting, broad range bactericides suitable for a variety of applications.

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Description

Nisin Compositions For Use As Enhanced, Broad Range Bactericides

Background of the Invention

This is a continuation-in-part application of Serial No. 209,861 filed June 22, 1988. Nisin is a polypeptide with antimicrobial properties which is produced in nature by various strains of the bacterium Streptococcus lactis. It is a known food preservative which inhibits the outgrowth of spores of certain species of Gram positive Bacilli.

Although sometimes mistakenly and imprecisely referred to as an antibiotic, nisin is more correctly classified as a bacteriocin, i.e. a proteinaceous substance produced by bacteria and which has antibacterial activity only towards species closely related to the species of its origin. Nisin is a naturally-occurring preservative found in low concentration in milk and cheese, and is believed to be completely non-toxic and non-allergenic to humans.

Nisin has recently been recognized as safe by the FDA as a direct food ingredient in pasteurized cheese spread, pasteurized processed cheese spread, and pasteurized or pasteurized processed cheese spread with fruits, vegetables, or meats. Furthermore, since it is a polypeptide, any nisin residues remaining in foods are quickly digested.

A summary of nisin's properties appears in Hurst, A., Advances in Applied Microbiology 27:85-123 (1981). This publication describes what is generally known about nisin. Nisin, produced by Streptococcus lactis, is available commercially as an impure preparation, Nisaplin™,

from Aplin & Barrett Ltd., Dorset, England and can be obtained by isolating naturally-occurring nisin from cultures of Streptococcus lactis and then concentrating the nisin according to known methods. There are also reported methods for producing nisin using altered strains of Streptococcus. See Gonzalez et al., U.S. Pat. No. 4,716,115, issued December 29, 1987. It should also be possible to produce nisin by recombinant DNA technology.

Nisin has been applied effectively as a preservative in dairy products, such as processed cheese, cream and milk. The use of nisin in processed cheese products has been the subject of recent patents. See U.S. Pat. Nos. 4,584,199 and 4,597,972. The use of nisin to inhibit the growth of certain Gram positive bacteria has been well documented. However, its complete success and acceptance as a food preservative has heretofore been hampered by the belief that nisin was ineffective against Gram negative and many Gram positive bacteria. Gram negative bacteria are almost always present in conjunction with Gram positive bacteria and are a major source of food spoilage and contamination. See Taylor, U.S. Pat. No. 5,584,199, issued April 22, 1986 and Taylor, U.S. Pat. No. 4,597,972, issued July 1, 1986; Tsai and Sandine, "Conjugal Transfer of Nisin Plasmid Genes from Streptococus Lactis 7962 to Leuconostoc Dextranicum 181, Applied and Environmental Microbiology, Feb. 1987, p. 352; "A Natural Preservative," Food Engineering Int'l,, May 1987, pp. 37-38; "Focus on Nisin," Food Manufacture, March 1987, p. 63.

Summary of the Invention

It has now been found that contrary to prior teaching, compositions comprising nisin, in combination with various non-bactericidal agents have enhanced, broad range bactericidal activity against Gram negative bacteria as well as enhanced activity against a broader range of Gram posi-

tive bacteria than nisin alone. The enhanced bactericidal activity against Gram positive bacteria occurs in a pH range broader than previously taught. The invention provides bacteriocin compositions of nisin or other, lanthionine containing bacteriocins, in combination with various non-bactericidal agents for example chelating agents or surfactants. The invention further provides the compositions dissolved or suspended in a suitable carrier to yield enhanced broad range bactericides.

Detailed Description of the Invention

Specifically, it has been found that a solution of about 0.1μg/ml to 300μg/ml of nisin in the presence of about 0.1 mM to 20 mM of a chelating agent, for example EDTA, virtually eliminates the growth of Gram negative bacteria such as Salmonella typhimurium, Escherichia coli, Pseudomonas aeruginosa, Bacterioides gingivalis, Actinobacillus actinomycetescomitans, and Klebsiella pneumoniae and is more active towards Gram positive bacteria such as Staphylococcus aureus, Streptococcus mutans, Listeria monocytogenes Streptococcus agalactiae and Coryneform bacteria than nisin alone. Although the enhancement of nisin activity by chelator was concentration dependent, contrary to expectations, concentrations of EDTA in excess of 20mM were inhibitory to the bactericidal activity of nisin. However, in the presence of a proteinaceous carrier, and polyvalent polymers such as serum albumin, collagen, gelatin, casein and keratin, the inhibition of nisin by concentrations of EDTA above 20mM was significantly reduced, thereby extending the useful range of EDTA enhancement of nisin.

It has also been found that a solution of about 0.1μg/ml to 300μg/ml nisin and about 0.1 mM to 20 mM of a chelating agent will further enhance the effectiveness of nisin against Gram negative and Gram positive bacteria in

the presence of about 0.01% to 1.0% of surfactant. Additionally, it has been found that, in the presence of surfactant alone, nisin has enhanced activity against Gram positive bacteria.

In the present invention, suitable chelating agents include, but are not limited to, EDTA, CaEDTA, $CaNa_2EDTA$, and other alkyldiamine tetraacetates, EGTA and citrate. Surfactants, valuable as cleansing agents, suitable for combination with nisin, with or without EDTA, include, but are not limited to, the nonionic surfactants Tweens, Tritons, and glycerides, ionic surfactants such as fatty acids, quaternary compounds, anionic surfactants such as sodium dodecyl sulphate and amphoteric surfactants such as cocamidopropyl betaine and emulsifiers.

Since Gram positive and Gram negative bacteria are almost always found together in foods, the effectiveness of the nisin compositions towards Gram negative bacteria such as Salmonella typhimurium, Escherichia Coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacterioides gingivalis, Actinobacillus actinomycetescomitans, and other Gram negative pathogens and Gram positive bacteria will be of great use. The bactericides are particularly suited for the control and prevention of contamination of raw ingredients, processed foods and beverages by bacterial pathogens and other microbial spoilage organisms. Potential food related uses include treatment of meats, especially poultry, eggs, cheese and fish and treatment of food packaging and handling equipment. Further uses include as food preservative, such as in processed cheese, cream, milk, dairy products and in cleaning poultry, fish, meats, vegetables, and dairy and food processing equipment. The use of the nisin compositions should not be limited to food related uses and the nisin compositions should be useful in any situation in which there is a need or desire to eliminate Gram negative and Gram positive bacteria.

The compositions can be dissolved in a suitable carrier for example an aqueous solvent or buffer or suspended in any suitable liquid, colloidal or polymeric matrix to create bactericides. The compositions or bactericides can be incorporated into ointments or coatings for medicinal uses such as the treatment of infections, wound dressings or surgical implants and as a broad spectrum disinfectant for skin or oral rinses, disinfectant scrubs, wipes or lotions. The bactericides can be used for cleaning medical instruments, in pre-operative surgical scrubs and the like. The bactericides are particularly useful in circumstances where environmental disinfection is desired but where chemical germicidals are precluded because of the risks of corrosive or otherwise toxic residues.

Unlike the activity of most broad spectrum germicidals which is compromised by the presence of complex organic matter, the compositions of the present invention are effective as bactericides in the presence of organic matter, such as milk or serum.

Nisin was known to optimally inhibit the growth of a few closely related Gram positive bacteria, particularly certain Gram positive spore forming bacilli at pH 5.0. The bactericidal activity of nisin in solution with a chelating agent was surprisingly rapid and greatly enhanced towards a broad range of Gram positive bacteria at pH values greater than pH 5.0, and, moreover, was activated towards Gram negative bacteria at both acidic and basic pH, preferably in the range pH 5.0 to 8.0. This unexpectedly rapid and broad-ranged bactericidal activity of chelator-activated nisin makes it suitable for use as, among other things, a disinfectant.

Nisin belongs to the class of peptide bacteriocins containing lanthionine. Also included among that class are subtilin, epidermin, cinnamycin, duramycin, ancovenin and Pep 5. These bacteriocin peptides are each produced by

different microorganisms. However, subtilin obtained from certain cultures of Bacillus subtilis, and epidermin obtained from certain cultures of Staphylococcus epidermidis, have been found to have molecular structures very similar to that of nisin (see Hurst, pp. 85-86, and Schnell et al., Nature, 333:276-278). It is therefore believed that because of the molecular similarities, other lanthionine containing peptide bacteriocins will be equally as effective as nisin in combination with chelating agents and non-ionic surfactants in eliminating Gram negative and Gram positive bacterial contaminations.

The effectiveness of the nisin, and by extension other lanthionine containing peptide bacteriocin, compositions as bactericides against Gram negative bacteria is surprising, since the prior art generally teaches away from this activity of nisin. The enhanced activity of nisin against Gram positive bacteria in the presence of EDTA at a pH greater than 5.0 is unexpected since it was previously believed that nisin activity is optimal at pH 5.0. Furthermore, the discovery of such effectiveness of the nisin and lanthionine containing peptide bacteriocin compositions as bactericides fulfills a long-felt need in the science of food preservation, which has suffered from the absence of an acceptable, natural, non-toxic agent effective against a broad range of bacteria.

In order to demonstrate the superior and unexpected rapid activity of the composition containing nisin, EDTA and/or various surfactants against both Gram negative and Gram positive bacteria, a number of experiments were conducted with the bactericides. These experiments are meant as illustration and are not intended to limit this invention. It is to be expected that other, lanthionine containing peptide bacteriocins would be effective substitutes for nisin and that chelating agents other than EDTA will be effective substitutes for EDTA.

All tests in the following examples were performed at 37°C. The efficacy of the enhanced broad range bactericides was determined by assaying bactericidal activity as measured by the percent bacterial survival after treatment with the bactericide. Generally, after incubation of a 10^7 cell per ml suspension of target species with the novel bactericide for specified lengths of time, bacteria were collected by centrifugation for 2 minutes. The bacterial pellet was washed free of the bactericide with a rescue buffer, termed herein Phage buffer (50mM Tris-HCl buffer pH 7.8, 1mM $MgSO_4$, 4mM $CaCl_2$, 0.1 M Nacl, and 0.1% gelatin), resuspended and serially diluted into Phage buffer, and 100ml of the suspended bacteria were spread on nutrient agar plates. Surviving bacteria were determined by scoring colony forming units (CFU) after incubation for 24-48 hours at 37°C. An effective bactericide according to this invention is one which allows less than 0.1% of the initial viable count of the bacteria to survive.

Example 1

Activity of Nisin and a Chelating Agent Against Gram Negative Bacteria (S. typhimurium)

As shown in Table 1, two tests were conducted in 20mM Tris, pH 8.0 at 37°C to show the effect of the bactericide containing nisin and the chelating agent EDTA alone. Test #1, a control, was conducted without EDTA and shows the effect of nisin alone toward the Gram negative bacterium S. typhimurium. The increased concentrations of nisin do exhibit some activity, but even the activity of the higher concentrations in the absence of EDTA, 1.6% survival at 100 μg/ml nisin, is wholly inadequate for a food preservative. The level of bactericidal activity obtained from nisin and EDTA is significant.

TABLE 1

Test #	Initial Viable Bacteria Count	EDTA (mM)	Nisin (μg/ml) 0	10	30	50	100	300
			Percentage S. typhimurium Survival at 3 hours					
1	3.0×10^6	0	100	51.3	-	7.0	1.6	-
2	5.7×10^6	20	2.5	-	10^{-3}	-	$<10^{-4}$	$<10^{-4}$

Test #2 (Table 1), conducted using nisin plus 20mM EDTA, demonstrates the surprising activity of the nisin composition in eliminating the target Gram negative bacteria.

Table 1 shows that in test #2 at a concentration of 20mM EDTA and 30 μg/ml of nisin, the bactericide has a marked bactericidal activity towards S. typhimurium, while at nisin concentrations of 100 μg/ml and greater, the nisin and EDTA bactericide virtually eliminates the bacteria (percentage survival less than 10^{-4} which indicates no surviving bacteria in the assay). Thus, the combination of EDTA and nisin demonstrates a synergistic activity of greater than 1000 times that of nisin alone.

Example 2

Activity of Nisin, a Chelating Agent and a Surfactant Against Gram Negative Bacteria (S. typhimurium)

Four tests (Table 2) were conducted to determine the effect on S. typhimurium of the bactericide containing nisin and both EDTA and the surfactant Triton X-100 in 20mM Tris, pH 8.0 at 37°. The control (Test #1) is identical to the control of Example 1 (Table 1).

TABLE 2

Test #	Initial Viable Bacteria Count	EDTA (mM)	Triton X-100 (%)	Nisin (g/ml) 0	10	30	50	100	300
				Percentage S. typhimurium Survival at 3 hours					
1	3.0×10^6	0	0	100	51.3	-	7.0	1.6	-
2	3.0×10^6	0	1.0	37.4	93.0	-	64.0	47.0	-
3	5.7×10^6	20	0.1	0.03	-	$< 10^{-3}$	-	-	-
4	5.7×10^6	20	1.0	$< 10^{-4}$	-	$< 10^{-4}$	-	$< 10^{-4}$	$< 10^{-4}$

Test #2 (Table 2) was conducted using nisin and 1.0% Triton X-100, but without EDTA. The presence of the detergent alone inhibits the activity of the nisin towards the Gram negative bacteria and nisin was ineffective. However, in tests #3 and #4 (Table 2), which represent the invention, the presence of 20mM EDTA in combination with Triton X-100 is a bactericide which markedly increases the bactericidal activity of nisin towards S. typhimurium. Indeed the combination of Triton X-100 with EDTA but without nisin was effective, although to a lesser degree than in the presence of nisin. While in both tests #3 and #4 (Table 2) the nisin combinations were very effective, the concentration of 1.0% Triton X-100 (test #4, Table 2) was most effective.

The presence of the non-ionic surfactant, Triton X-100, in combination with EDTA, enhances the activity of nisin toward Gram negative bacteria even more than the bactericide containing nisin and EDTA alone (Example 1).

Example 3

Activity of Nisin, a Chelating Agent and a Surfactant Against Gram Negative Bacteria (S. typhimurium)

Table 3 shows the enhanced activity toward S. typhimurium of the bactericide containing nisin, 20mM of the chelating agent EDTA and the non-ionic surfactant Tween 20 in 20 mM Tris, pH 8.0 at 37°C. As with Triton X-100 (Example 2) the combination of nisin and EDTA with (1%) of Tween 20 is most effective.

TABLE 3

Test #	Initial Viable Bacteria Count	EDTA (mM)	Tween20 (%)	Nisin (μg/ml) 0	10	30	50	100	300
				Percentage S. typhimurium Survival at 3 hours					
1	3.0×10^6	0	0	100	51.3	-	7.0	1.6	-
2	5.7×10^6	20	0	2.5	-	$< 10^{-3}$	-	$< 10^{-4}$	$< 10^{-4}$
3	4.3×10^6	20	1.0	$< 10^{-2}$	-	$< 10^{-4}$	-	$< 10^{-4}$	$< 10^{-4}$

Example 4

Activity of Nisin, a Chelating Agent and a Surfactant Against Gram Negative Bacteria (Escherichia coli)

The effect of the bactericide containing nisin and EDTA towards the Gram negative bacteria E. coli was demonstrated, as shown in Table 4.

TABLE 4

Test #	Initial Viable Bacteria Count	Triton EDTA (mM)	X-100 (%)	Nisin (μg/ml) 0	30	100	300
				Percentage E. coli Survival at 2 hours			
1	1.0×10^7	0	0	100	27	25	8.5
2	1.0×10^7	20	0	14.5	0.86	0.01	0.001
3	1.0×10^7	0	1.0	100	-	30	-
4	1.0×10^7	20	1.0	1.2	0.8	0.05	$<10^{-4}$

The tests, with and without EDTA, were performed in 20mM Tris buffer solution, pH 8.0 at 37°C, with an initial viable count of 1×10^7 E. coli cells/ml. The effects of the bactericide were measured as a function of percentage bacteria survival after 2 hours.

In test #1, (control, Table 4) without EDTA, nisin exhibited little meaningful activity toward the elimination of E. coli. In test #2 (Table 4), however, where 20mM EDTA was present, the bactericidal composition exhibited substantial activity towards the E. coli bacteria. The activity increased in effectiveness as the concentration of nisin was increased. The combination of nisin with EDTA as a bactericide demonstrates a 1000 fold synergistic increase in effectiveness towards E. coli. In tests #3 and #4 (Table 4), it can be seen that Triton X-100 has no significant bactericidal activity towards E. coli. In fact, Triton X-100 appears to inhibit nisin activity towards Gram negative bacteria as was found with S. typhimurium (Table 2). However, the overall enhancement of nisin by EDTA substantially reverses the inhibitory effects of Triton X-100 as seen in Tables 2 and 4.

It thus appears that the bactericide containing nisin and a chelating agent, such as EDTA, is an effective food preservative towards various types of Gram negative bacteria even in the presence of surfactants.

Example 5

Activity of Nisin and a Chelating Agent Against Gram Negative Bacteria (Klebsiella pneumoniae)

The effect of the bactericide containing nisin and EDTA alone towards the Gram negative bacteria K. pneumoniae was demonstrated, as shown in Table 5.

TABLE 5

Test	Initial Viable Bacteria Count	EDTA (mM)	Triton X-100 (%)	Nisin μg/ml 0	30	100	300
				% Survival at 2 hours			
1	10^7	0	0	100	-	50	38
2	10^7	20	0	22	0.5	1.1	0.085

The two tests, one with and one without EDTA (control), were performed in 20 mM Tris buffer, pH 8.0 at 37°C with an initial viable count of 10^7 cells/ml of K. pneumoniae. The effect was measured as a function of percentage bacterial survival after 2 hours.

In test #1, (control, Table 5) without EDTA, nisin exhibited little meaningful bactericidal activity toward K. pneumoniae. In test #2 (Table 5), however, where 20mM EDTA was present, the bactericide exhibited substantial activity towards K. pneumoniae. The activity increased in effectiveness as the concentration of nisin was increased.

Example 6

Nisin Activity Against Gram Negative Bacteria (Salmonella typhimurium) is Dependent on Chelator Concentration

The data in Table 6 demonstrate that the enhanced activation of nisin towards Gram negative bacteria (S. typhimurium) is dependent on the concentration of EDTA in either 50 mM sodium acetate, pH 5.0, or 20 mM Tris, pH 8.0 at 37°C.

TABLE 6

Test #	pH	Initial Viable Bacterial Count	Nisin 4g/ml	EDTA(mM) 0	0.2	2.0	10	50	100
				% Survival at 2 hours					
1	5.0	$3x10^6$	0	100	-	38.7	15.2	3.5	-
2	5.0	$3x10^6$	100	0.6	10^{-4}	10^{-4}	0.004	0.02	-
3	8.0	$5x10^6$	0	100	-	8.7	14	11.4	45
4	8.0	$5x10^6$	100	4	10^{-4}	10^{-4}	10^{-4}	0.6	30

In tests #1 and #3, (controls, Table 6) using EDTA concentrations up to 100 mM without nisin exhibited little meaningful activity towards S. typhimurium at either pH 5.0 (#1) or pH 8.0 (#3). In tests #2 and #4 (Table 6), however, where 100 μg/ml nisin was present in combination with EDTA, the bactericides exhibited substantial activity towards S. typhimurium. The activity of the bactericides was similar at both acidic pH (5.0) and basic pH (8.0), despite the fact that the activity of nisin alone towards Gram positive bacteria is optimal at pH 5.0.

The enhancement of nisin by EDTA was concentration dependent, being optimal in the range 0.2 mM to 10 mM at pH values 5.0 and 8.0. Surprisingly, at concentrations greater than 10 mM EDTA, the enhancement of nisin by EDTA becomes reduced; the reduction of activation is significantly greater at pH 8.0 than at pH 5.0.

Example 7

Nisin and a Chelating Agent Against Gram Negative Bacteria (S. typhimurium)

The enhancement of the activity of nisin by EDTA towards Gram negative bacteria in the presence of biological tissue was demonstrated with S. typhimurium on chicken muscle, and is shown in Table 7.

TABLE 7

Test #	pH	Nisin μg/ml	EDTA(mM) 0	0.1	0.3	1	3	10	20	30	100
			% Survival[a] at 2 hours								
1	5.0	0	11.8	-	-	-	-	6.4	-	-	-
2	5.0	300	0.1	0.2	0.05	0.01	0.003	0.016	0.03	0.02	0.07
3	8.0	0	100	-	-	-	-	5.2	-	-	-
4	8.0	300	7.5	0.1	0.02	0.02	0.09	0.47	0.5	-	2.2
5	8.0	300[b]	0.02	0.09	0.0002	$<10^{-4}$	0.0004	0.003	-	0.03	0.09

[a] Unadhered cells

[b] Contains 1% Bovine serum albumin (BSA)

Incubations were performed in either 50mM sodium acetate, pH 5.0, or 20 mM Tris, pH 8.0 at 37°C.

Cubes of chicken muscle were cleansed with sodium hypochlorite and povidone iodine prior to use. To inoculate the tissue, the cubes of chicken muscle were dipped into a 10^8 cells/ml suspension of S. typhimurium in 20 mM Tris HCl, pH 8.0. Excess moisture was removed from dipped cubes by tapping. The chicken samples were placed into sufficient buffer containing the nisin composition to cover the tissue and incubated for 2 hours at 37°C after which the tissue was removed to sufficient Phage buffer to cover the tissue. The bacteria remaining in the test solution were collected by centrifugation, washed with Phage buffer, and combined with bacteria washed from the tissue by Phage buffer. The combined samples (termed "unadhered" cells) were serially diluted and 100µl aliquots were plated for determination of surviving bacteria.

In tests #1 and #3 (Table 7), in the absence of nisin at either pH 5 or pH 8, EDTA alone has no significant effect on the survival of S. typhimurium. In tests #2 and #4 (Table 7), however, where 300 µg/ml nisin was present, the bactericides exhibited substantial activity towards S. typhimurium on chicken muscle at both pH 5.0 and pH 8.0.

The enhancement of nisin by EDTA was concentration dependent, the optimal concentration being in the range 0.3mM to 10mM EDTA at both pH values 5.0 and 8.0. At concentrations greater than 10mM EDTA at pH 8.0, the activation of nisin by EDTA is reduced. However, as is shown in test #5 (Table 7), in the presence of 1.0 % bovine serum albumin at pH 8.0, the efficacy of nisin towards S. typhimurium on chicken muscle is expressed throughout the range of EDTA concentrations up to 100mM.

Thus, bactericides containing nisin and low concentrations of chelating agent, such as EDTA in the range

0.1mM to 20mM, can be extremely effective for the elimination or prevention of contamination of food by Gram negative bacteria.

Example 8

Titration of Nisin Activity Against Gram Negative Bacteria (S. typhimurium)

At the optimal concentration of chelating agent, the efficacy of the bactericide in Tris buffer towards Gram negative bacteria was demonstrated to be substantial, as is shown in Table 8.

TABLE 8

Test #	Initial Viable Bacterial Count	EDTA (mM)	BSA %	Nisin μg/ml 0	0.1	0.3	1.0	3.0	10	30	100
				% Survival at 2 hours							
1	$6x10^6$	0	0	100	-	-	-	-	51.3	-	1.6
2	$6x10^6$	1.0	1.0	63	0.7	0.08	0.01	0.05	0.01	$<10^{-4}$	-

In test #2 (Table 8), it can be seen that as little as 0.3 μg/ml of nisin, with 1.0 mM EDTA in 20mM Tris at pH 8.0 in the presence of 1% bovine serum albumin (BSA), significantly reduced the survival of S. typhimurium. The bactericide is as active towards Gram negative bacteria as nisin alone is towards Gram positive Streptococci.

Example 9

Titration of Nisin Activity Against Gram Negative Bacteria (S. typhimurium)

At the optimal concentration of chelating agent, the efficacy of a bactericide towards Gram negative bacteria in the presence of biological tissue was demonstrated with S. typhimurium on chicken muscle, and is shown in Table 9.

TABLE 9

Test #	pH	Initial Viable Bacterial Count	EDTA (mM)	BSA (%)	Nisin μg/ml 0	10	100	200	300
					% Survival at 2 hours				
1	8.0	$3x10^7$	0	0	100	-	-	-	-
2	8.0	$3x10^7$	1.0	1.0	27	0.26	0.008	0.007	0.006

Cubes of chicken muscle were cleansed with sodium hypochlorite and povidone iodine prior to use. To inoculate the tissue, the cubes of chicken muscle were dipped into a 10^8 cells/ml suspension of S. typhimurium in 20 mM Tris HCl, pH 8.0. Excess moisture was removed from dipped cubes by tapping. The tissue was placed into sufficient buffer containing the nisin compositions to cover the tissue, and incubated for 2 hours at 37°C after which the tissue was removed to sufficient Phage buffer to cover the tissue. The bacteria remaining in the test solution were collected by

centrifugation, washed with Phage buffer, and combined with bacteria washed from the tissue by Phage buffer. The combined samples (termed "unadhered" cells) were serially diluted and 100µl aliquots were plated for determination of surviving bacteria.

Example 10

Nisin EDTA and Methyl Paraben Activity Against Gram Negative Bacteria (S. typhimurium)

A bactericide containing nisin and EDTA, when combined with a known food preservative, methyl paraben, was demonstrated to be exceptionally effective towards Gram negative bacteria, as shown in Table 10.

TABLE 10

Test #	Initial Viable Bacterial Count	Nisin g/ml	EDTA[b] (mM)	% Methyl Paraben 0	0.1	1.0
				% Survival[c] at 2 hours		
1	$3x10^6$	0	10	11.8	1.0	10^{-4}
2	$3x10^6$	300	10	0.03	$<10^{-3}$	$<10^{-4}$

[b] 50 mM Na acetate buffer, pH 5.0

[c] Unadhered cells

Cubes of chicken muscle were cleaned with sodium hypochlorite and povidone iodine prior to use. To inoculate the tissue, the cubes of chicken muscle were dipped into a 10^8 cells/ml suspension of S. typhimurium in 50 mM sodium acetate buffer, pH 5.0. Excess moisture was removed from dipped cubes by tapping. The tissue was placed into sufficient buffer containing nisin compositions to cover the tissue, and incubated for 2 hours at 37°C after which the

tissue was removed to sufficient Phage buffer to cover the tissue. The bacteria remaining in the test solution were collected by centrifugation, washed with Phage buffer, and combined with bacteria washed from the tissue by Phage buffer. The combined samples (termed "unadhered" cells) were serially diluted and 100 µl aliquots were plated for determination of surviving bacteria.

In test #1 (Table 10), methyl paraben in the presence of 10 mM EDTA was shown to be effective towards S. typhimurium only at a concentration of 1.0%. In test #2 (Table 10), however, in the presence of 300 µg/ml nisin, the effectiveness of methyl paraben and nisin towards S. typhimurium was substantially improved.

The compositions containing nisin and EDTA significantly improve the utility of the food preservative methyl paraben. Furthermore, the bactericides may lead to substantial reductions in the concentrations, or eliminate the need for these commonly recognized, though less desirable, food preservatives such as methyl paraben.

Example 11

Nisin and Chelating Agent Activity Against Gram Positive Bacteria (Staphylococcus aureus)

The activation of nisin by a chelating agent is pH-dependent. The data in Table 11 confirm that at pH 5.0, nisin is somewhat more bactericidal towards S. aureus than is nisin at pH 8.0. At pH 5.0, EDTA does not enhance nisin activity towards S. aureus and at concentrations of EDTA greater than 10 mM, EDTA is inhibitory to the bactericidal activity of nisin. However, the bactericidal activity of nisin activated by EDTA at pH 8.0 is significantly greater than the bactericidal activity of nisin alone, or in combination with EDTA at pH 5.0.

Table 11

Influence of pH on the Effects of EDTA on Nisin Bactericidal Activity towards Staphyloccus aureus

pH	Nisin μg/ml	EDTA mM 0	0.1	0.3	1.0	3.0	10	30	100
		% Survival 2 hr[a]							
8.0	0	100	-	100	81	100	100	100	-
8.0	3.0	7.4	0.03	0.01	0.2	0.4	3	56	-
5.0	0	100	-	-	-	100	-	-	-
5.0	3.0	0.6	I.0	1.3	1.4	1.8	-	34	80

[a] Initial viable count: 8.0×10^6 cfu/ml

Incubations were performed in 50 mM sodium acetate buffer, pH 5.0 or 20 mM Tris-HCl buffer, pH 8.0 at 37°C.

The bactericidal activity of nisin alone is reported (see Hurst) to be greatest at pH 5.0 or lower, and data presented in Table 11 support this. On the basis of this information it was believed that the bactericidal activation of nisin by EDTA towards S. aureus would likewise be greatest at lower pH. However, as can be seen in Table 11 and contrary to expectations (see Table 6), EDTA was not observed to enhance nisin activity towards Gram positive bacteria at pH 5.0. However, inhibition of nisin activity by high concentrations of EDTA was still observed at pH 5.0. Thus, the activation of nisin by a chelating agent occurs only within a range of chelator concentrations and, with respect to Gram positive bacteria, is dependent upon pH with the preferred pH range greater than pH 5.0.

Example 12

Nisin and Chelating Agent Activity Against Gram Positive Bacteria

The effects of EDTA on the bactericidal activity of nisin at pH 8.0 are not limited to S. aureus, an important human pathogen, but are also observed with Streptococcus mutans, responsible for dental plaque (Table 12A), Listeria monocytogenes, a foodborne pathogen (Table 12B), and with a mixed population of axillary Coryneform bacteria, contributors to body odor (Table 12C).

Table 12A

The Effects of EDTA on the Bactericidal Activity of Nisin towards Streptococcus mutans

pH	Nisin μg/ml	EDTA mM 0	0.01	0.1	0.3	1.0	3.0	10	30	100
		% Survival after 2 hr[a]								
8.0	0	100	-	-	-	-	-	-	-	-
8.0	0.1	4.3	1.8	0.04	0.02	0.06	1	25	100	100

[a] Initial viable count: 6.0×10^6 cfu/ml

Incubations were performed in 20 mM Tris-HCl, pH 8.0 at 37°C.

Table 12B

The Effects of EDTA on the Bactericidal Activity towards Listeria monocytogenes

pH	Nisin μg/ml	EDTA mM 0	0.1	0.3	1.0	3.0	10	30	100
		% Survival after 2hr[a]							
8.0	0	100	-	-	84	-	-	-	-
8.0	3.0	0.71	0.04	0.04	0.02	0.1	0.64	10	14

[a] Initial viable count: 6.0×10^6 cfu/ml

Incubations were performed in 20 mM Tris-HCl, pH 8.0 at 37°C.

Table 12C

The Effects of EDTA on Nisin Bactericidal Activity towards Coryneform bacteria

pH	Nisin g/ml	EDTA mM 0	0.1	0.3	1.0	3.0	10
		% Survival 2hr[a]					
8.0	0	100	-	4.6	3.6	8	36
8.0	3	0.22	0.03	0.0009	0.1	--	0.16

[a] Initial viable count: 1.0×10^6 cfu/ml

Incubations were performed in 20 mM Tris-HCl, pH 8.0 at 37°C.

Example 13

Rapid Bactericidal Activity of Nisin Activated by Chelator

The bactericide comprising nisin with EDTA is rapidly bactericidal as is illustrated by the data presented in Table 13A. Suspensions of the Gram positive bacterium S. mutans at 10^7 cells/ml were incubated in 20 mM Tris buffer, pH 7.3 at 37°C with a range of concentrations of nisin activated by 1 mM EDTA. The suspensions were incubated for various times ranging from 0.5 to 60 minutes with the bactericides. The bactericidal efficacy of the bactericides was estimated by determining the percent survival of bacteria. Enhanced by EDTA, as little as 10 μg/ml of the nisin in this formulation is able to reduce the bacterial load by 6 logs within 1 minute.

Rapid bactericidal activity is a prerequisite for effective disinfection. Thus, the compositions are predicted to be effective bactericides particularly as demonstrated here, as a component of a mouthwash, rinse, toothpaste, or other similar dentrifice active against plaque forming S. mutans.

The activity of nisin enhanced by EDTA against Gram negative bacteria after 2-3 hours was shown in Examples 1-7. Rapid bactericidal activity of nisin enhanced by EDTA is also seen towards Gram negative bacteria and this is illustrated by the data in Table 13B.

Table 13A

Kinetics of Bactericidal Activity towards Streptococcus mutans of Nisin Enhanced by EDTA

Incubation Time (Minutes)	Nisin μg/ml with 1.0 mM EDTA					
	0	1	3	10	30	100
	% Survival[a]					
0.5	-	-	-	-	-	$<10^{-4}$
1	-	-	-	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
3	100	0.5	0.002	$<10^{-4}$	$<10^{-4}$	-
15	-	0.03	$<10^{-4}$	$<10^{-4}$	-	-
30	-	-	$<10^{-4}$	-	-	-
60	100	0.003	-	-	-	-

[a] Control viable cell count: 1.0×10^{7} cfu/ml

Incubations were performed in 20 mM Tris-HCl, pH 7.3 at 37°C.

TABLE 13B

Rapid Bactericidal Activity towards Escherichia coli of Nisin Enhanced by EDTA

	Nisin μg/ml						
mM EDTA	0	0.3	1.0	3	10	30	100
	% survival at 1 min[a]						
1.0	100	100	56	0.37	0.013	0.015	0.008

[a] Initial viable count: 1.0×10^7 cfu/ml

Incubations were performed in 20 mM Tris, pH 7.0 at 37°C.

Example 14

Effect of Divalent Cations on EDTA Enhancement of Nisin Activity

Divalent cations bind to EDTA and other chelating agents and would be expected to neutralize the activation of nisin by EDTA. However, as can be seen by the data in Table 14, the bactericidal activity of nisin against S. mutans is enhanced by 1 mM EDTA even in the presence of 1 mM Ca^{2+} ion; only above 3 mM was Ca^{2+} ion inhibitory to EDTA-activated nisin. This is particularly important in mouthwash applications where calcium ion concentrations are relevant.

TABLE 14

Rapid Bactericidal Activity towards Streptococcus mutans of Nisin Activated by EDTA in the presence of Divalent Cation

Nisin	$CaCl_3$ mM 0	0.1	0.3	1.0	3	10
	% survival at 1 min.[a]					
0	100					
3	2.9					
3[E]	0.0042	0.0042	0.052			18
30[E]	0.0019		0.0003	0.0004	0.06	6.8
100[E]	$<10^{-4}$		$<10^{-4}$	$<10^{-4}$	0.0001	1.5

[E] 1 mM Na_2EDTA

[a] Initial viable count 1.0 X 10^2 cfu/ml.

Incubations performed in 10% Fetal Calf Serum at 37°C.

Example 15

Nisin and Surfactant Activity Against Gram Positive Bacteria

The bactericidal activity of nisin can also be significantly enhanced when combined with a surfactant alone. This is best illustrated at a limiting nisin concentration (0.2 μg/ml) as shown in Table 15A. At concentrations up to 0.1%, the food grade surfactant monolaurin has little significant bactericidal activity towards Streptococcus agalactiae in the complex medium milk. Nisin, at concentrations up to 0.2 μg/ml, likewise does not

exhibit significant bactericidal activity in milk. However, the combination of the two agents, 0.1% monolaurin and nisin 0.2 g/ml, is extremely potent towards S. agalactiae. This bactericide is over 100 times more active than what would be expected for the additive effect and 10,000 times more active than either of the components individually. Thus, when the application of nisin is limited by its available activity, a bactericide comprising nisin with a surfactant can be expected to be more useful.

An example of where the application of nisin is limited by its available activity is illustrated by the data in Table 15B. Although nisin, and particularly the bactericide comprising nisin and EDTA, is bactericidal towards L. monocytogenes, the data in Table 15B demonstrate that in a complex medium like milk the available nisin activity towards this organism is restricted. However, the bactericide comprised of nisin with the glyceride, monooleate, is effective in milk towards this foodborne pathogen even though monooleate by itself had no bactericidal activity towards this organism.

Table 15A

Nisin Bactericidal Activity towards Streptococcus agalactiae in milk at 37°C

(Activation of nisin by monolaurin)

Nisin (μg/ml)	Monolaurin (%)		
	0	0.01	0.1
	% survival at 2h[a]		
0	100	100	4.5
0.02	100	100	0.2
0.2	2.2	0.05	0.0008

[a] Initial visable counts 6.0×10^7 cfu/ml.

Incubations were in milk at 37°C.

Table 15B

Nisin Bactericidal Activity towards Listeria monocytogenes in milk at 37°C

(Activation of nisin by monooleate)

Nisin μg/ml	% Monooleate 0	0.1	1.0
	% Survival 2 hr[a]		
0	100	67	63
100	0.56	10^{-3}	10^{-4}

[a] Initial viable count 5.0×10^7 cfu/ml

Incubations were in milk at 37°C.

Claims

1. A composition comprising a lanthionine containing bacteriocin and a chelating agent.

2. A composition comprising a lanthionine containing bacteriocin and a surfactant.

3. A composition comprising a lanthionine containing bacteriocin, a chelating agent and a surfactant.

4. The composition as defined in claim 1, 2 or 3 wherein the lanthionine containing bacteriocin is selected from the group consisting of nisin, subtilin, epidermin, cinnamycin, duramycin, ancovenin and Pep 5.

5. The composition as defined in claim 1 or 3 wherein the chelating agent is selected from the group consisting of alkyldiamine tetraacetates, CaEDTA, $Na_2CaEDTA$, EGTA and citrate.

6. The composition as defined in claim 5 wherein the alkyldiamine tetraacetate is EDTA and the bacteriocin is nisin.

7. The composition as defined in claim 2 or 3 wherein the surfactant is selected from the group consisting of Tritons, Tweens, glycerides, fatty acids, emulsifiers, quaternary compounds, amphoteric and anionic surfactants.

8. The composition as defined in claim 1 also containing a food perservative.

9. An enhanced broad range bactericide comprising a carrier, a lanthionine containing bacteriocin and a chelating agent.

10. An enhanced broad range bactericide comprising a carrier and a lanthionine containing bacteriocin and a surfactant.

11. An enhanced broad range bactericide comprising a carrier, a lanthionine containing bacteriocin, a chelating agent and a surfactant.

12. The enhanced broad range bactericide as in claim 9, 10 or 11 wherein the lanthionine containing bacteriocin selected from the group consisting of nisin, subtilin, epidermin, cinnamycin, duramycin, ancovenin and Pep 5 and the chelating agent selected from the group consisting of alkyldiamine tetraacetates, EGTA and citrate are present in quantities such that the bactericide has enhanced effectiveness against at least one of the bacteria from the group consisting of Staphylococcus aureus, Streptococcus mutans, Listeria monocytogenes, Streptococcus agalactiae, Cornyeform bacteria, Salmonella typhimurium, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacterioides gingivalis and Actinobacillus actinomycetescomitans.

13. The enhanced broad range bactericide as in claim 12 wherein the alkyldiamine tetraacetate is EDTA.

14. The enhanced broad range bactericide as in claim 10 or 11 wherein the surfactant is selected from the group consisting of Tritons, Tweens, glycerides, fatty acids, quaternary compounds, emulsifiers, amphoteric and anionic surfactants and is present in an amount sufficient such that the bactericide has enhanced effectiveness against at least one of the bacteria from the group consisting of Gram negative and Gram positive bacteria.

15. The enhanced broad range bactericide as in claim 12 wherein the concentration of nisin is between about 0.1 μg/ml and 300.0 μg/ml and the concentration of chelating agent is between about 0.1 mM and 20mM.

16. The enhanced broad range bactericide as in claim 14 wherein the concentration of surfactant is between about 0.01% and 1.0%.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/02625

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: A 23 C 19/11, A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	A 23C, A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	GB, A, 738655 (APLIN & BARRETT LTD) 19 October 1955, see page 2, line 115 - page 3, line 28 --	1-16
A	Chemical Abstracts, vol. 86, no. 1, 3 January 1977, (Columbus, Ohio, US), A.I. Pedenko et al.: "Effect of the antibiotic nisin on pathogenic staphylococci and streptococci" see page 58, abstract no. 594x & Tr. S'ezda Mikrobiol. Ukr. 4th 1975, 221-2	1-16

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
2nd October 1989	24 OCT 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8902625 SA 29530

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 18/10/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB-A- 738655		None	

Search: (WO8912399)/PN/XPN

1 / 1 Patent Number: FI895878 D0 19891208

NISIN COMPOSITIONS FOR USE AS ENHANCED, BROAD RANGE BACTERICIDES.

(FI8905878)

Lantioninhaltiga bakteriocinkompositioner för användning som förbättrade baktericider med ett brett spektrum

NISIN-SAMMENSETNINGER ANVENDT SOM BREDSPEKTREDE BAKTERICIDER.

PRAEPARAT INDEHOLDENDE ET LANTHIONIN-HOLDIGT BACTERIOCIN OG ET CHELATERINGSMIDDEL

(EP-382814)

Bacteriocin compositions comprising lanthionine containing bacteriocins and non-bactericidal agents. When the bacteriocin compositions are combined with a suitable carrier with each component present in sufficient quantities such that the composition is effective against Gram negative bacteria in addition to Gram positive bacteria, they become enhanced, rapid acting, broad range bactericides suitable for a variety of applications.
(From WO8912399 A1)

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(A2) APPLIED MICROBIOLOGY INC (US)
TAY, TENG-TIOW; FROM 19910211 TO 19960502
AMBI, INC.; FROM 19910211 TO 19970811
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APPLIED MICROBIOLOGY; FROM 19921006
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(D1) APPLIED MICROBIOLOGY INC (US)
(D1) APPLIED MICROBIOLOGY INC (US)

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AP :	1989FI-0005878 19891208		
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STG:	Patent application filed		
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STG:	Translation of European patent specification
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STG:	Granted EP number in Bulletin
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STG:	Application of patent for invention
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STG:	Doc. laid open (First publication)
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STG:	Patent specification
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FD :	Technical priority: CS689789A 19891206 [1989CS-0006897]
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Designated States:
(WO8912399)
AU DK FI HU JP KR MC NO SU
European patent : AT BE CH DE FR GB IT LU NL SE